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Hepatitis B virus reactivation in HBsAg-negative patients is associated with emergence of viral strains with mutated HBsAg and reverse transcriptase

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ABSTRACT

Background/aims: Virological factors associated with hepatitis B virus reactivation (HBV-R), following chemotherapy for cancer in hepatitis B surface antigen (HBsAg)-negative patients, are not well known.
Materials and methods: HBV strains from 16 patients presenting HBV-R following chemotherapy were studied and compared to those obtained from 51 HBV chronically-infected patients.
Results: HBsAg variability was significantly increased within the major hydrophilic region, the a determinant and the C-terminal region. Amino acid substitutions were more frequently found in HBV-R patients as compared to controls at 17 and 11 positions within HBsAg and HBV-RT, respectively. This resulted in atypical serological testing in 56% of patients and detection of resistance mutation to nucleoside analogs in 12.5%.
Conclusion: HBsAg and HBV-RT mutations are frequently encountered in patients with HBV-R, resulting in atypical serological testing and emergence of HBV strains resistant to nucleos(t)ides analogs.

Introduction

Hepatitis B virus (HBV) reactivation is a rising concern in patients undergoing chemotherapy for cancer. Indeed, an increasing number of therapeutic agents used are likely to interfere with the natural course of HBV infection. Thus, HBV reactivation (HBV-R) has been described with the use of biotherapies such as monoclonal antibodies like rituximab (Lalazar et al., 2007; Ling et al., 2013). In this context, recommendations have been proposed for the management of HBV-infected patients necessitating chemotherapy for cancer and preventive treatment of hepatitis B surface antigen (HBsAg)-positive patients with nucleos(t)ides analogs is recommended (European Association For The Study Of The Liver, 2012). The risk of HBV reactivation is much lower in patients negative for HBsAg but positive for anti-hepatitis B core antibody (HBcAb) and the management of these patients is still debated (Hsu et al., 2014).

Clinical factors associated with HBV-R following chemotherapy in initially HBsAg-negative patients have been previously studied. Indeed, male sex, type of cancer including hematological or breast cancer, intensity of therapy, rituximab use and a low anti-HBsAb level at baseline have been reported to be associated with an increased risk for HBV-R (Hsu et al., 2014; Yeo et al., 2009; Seto et al., 2014; Mikulska et al., 2014; Kim and Kim, 2014). Specific HBV genotypic patterns might also be found in this clinical setting. Indeed, mutations in the basal core promoter conferring higher

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replication capacity have been reported in fulminant forms of HBV-R (Hsu et al., 2014; Borentain et al., 2010; Alexopoulou et al., 2006; Yeo et al., 2000; Gérolami et al., 2005), and mutations within HBsAg have been described in HBsAg-negative-patients presenting HBV reactivation, in some cases in association with rituximab administration (Hsu et al., 2014; Seto et al., 2014; Mikulska et al., 2014; Kim and Kim, 2014; Borentain et al., 2010; Westhoff et al., 2003; Sugauchi et al., 2011). However, HBV genotypic characteristics have not been extensively studied in patients undergoing HBV-R and the actual frequency of these genotypic patterns as well as their consequences in the management of these patients are not known.

In the present work we systematically studied genotypic patterns of HBV collected from 16 consecutive HBsAg-negative patients who developed HBV-R following chemotherapy in our institution. All these patients were HBsAg-negative but positive for anti-HBc with or without anti-HBsAb at time of initiation of CT. We show that in these patients, mutations within core/precore regions are systematically encountered in association with an increased variability within HBsAg and HBV reverse transcriptase (HBVrt). The emergence of such HBV mutants in this clinical context may have important implication in the management of these patients.

Statistical analysis

The data were analyzed using SPSS v9.0 software program. Pearson's chi-square test and Fisher's exact test were used for comparison of categorical variables, and the Student's *t*-test was used for comparison of continuous variables. Statistical significance was set at the 0.05 level.

Results

HBV serological/molecular status before initiation of chemotherapy and at time of reactivation

Results of serological patterns at time of diagnosis of reactivation and before chemotherapy initiation are presented in Table 2.

HBV status before chemotherapy initiation

Before chemotherapy initiation, all 16 patients were HBsAg-negative. None of these patients received prophylactic treatment. Anti-HBsAb could be detected in 10 of 15 (66 %) patients tested at baseline (mean titer, 114 IU/L (range, 14–897 IU/L)). HBV DNA determination could be retrospectively performed before chemotherapy in seven patients. HBV DNA was detected in 5 of them (71%) (Table 2). In one patient (pt 11), HBsAg was retrospectively found positive before chemotherapy using a second test (Vidas). HBV DNA determination could not be done before chemotherapy in this patient.

HBV status at time of reactivation

HBV reactivation occurred during chemotherapy in 5/16 patient, and in the 11/16 remaining patients between 3 and 12 months following the end of chemotherapy (mean 5.5 months). At time of reactivation, diagnosis of HBV reactivation was made difficult by atypical serological testing in 5 patients (31%; pts 2, 6, 10, 12, 16) (Table 2). Thus, at time of reactivation, HBsAg was found negative in three patients (pts 2, 6, 10) and HBsAg and anti-HBsAb were concurrently detected in two other patients (pts 12, 16). In patient 2, HBsAg could be retrospectively detected using the Vidas assay instead of the Axsym assay. Patients 6 and 10 were found negative for HBsAg using both Axsym and Vidas tests, while patient 10 presented with a serological pattern indicating cured HBV infection (HBsAg-negativity, anti-HBsAb-positivity (535 IU/L)). Finally, in all cases, diagnosis of HBV reactivation was definitively set by HBV DNA detection.

Overall, pitfalls in the serological diagnosis of HBV infection, before chemotherapy and/or during the course of anticancer therapy, could be demonstrated in 9 out of 16 patients (56%) (pts 2, 5, 6, 8, 10, 11, 12, 14, 16). In these 9 patients, systematic HBV DNA determination before CT and during follow-up might have led to introduce earlier anti-HBV therapy and possibly to modify the course of HBV reactivation. Indeed, one of these patients (pt 5), who was retrospectively found HBV DNA positive before CT, died from fulminant hepatic failure despite introduction of lamivudine therapy at time of clinically-symptomatic HBV reactivation.

Table 1
Clinical and virological characteristics of patients with HBV reactivation and controls.

	Patients with HBV reactivation at time of reactivation, $N=16$	Controls, $N=123$
Median age (years)	65	43
Gender (M/F)	15/1	79/44
HBV DNA detection	16/16	51/123
HBV DNA quantification		
> 10000 IU/mL	11/16	16/94
< 200 IU/mL	0/16	50/94
HBV genotype		
A	1/16	26/51
B	0/16	0/51
C	2/16	2/51
D	13/16	18/51
E	0/16	5/51
Non A	15/16	25/51

Outcome of patients following HBV reactivation

From 2002 through 2006, all patients received after the diagnosis of HBV reactivation either lamivudine ($n=7$), lamivudine plus adefovir ($n=1$) or no treatment ($n=1$). From 2006 through 2011, all patients received either entecavir ($n=4$) or tenofovir ($n=3$) as first line treatment. Four patients (25%) died from fulminant hepatic failure (pts 1, 3, 5 and 9). All of these patients were taken in charge before 2006 and had received either lamivudine ($n=3$) or no treatment ($n=1$), whereas no HBV-related death was observed during the 2006–2010 period ($p=0.04$).

Genotypic patterns of HBV from patients with reactivation

To identify specific HBV genotypic patterns associated with HBV reactivation, HBV sequences obtained from the 16 patients with HBV reactivation were compared with those obtained from the 51 newly diagnosed chronic HBV-carriers.

Genotype, precore and core promoter mutations

Patients presenting HBV reactivation were infected with genotypes D ($n=13$), C ($n=2$) and A ($n=1$). Non-A genotypes were significantly more frequent in HBV-R patients versus controls (94% vs. 49%; $p=0.012$).

Mutation A1762C within the core promoter region was detected in two patients infected with genotype D HBV (Table 3); this later mutation was never found in HBV sequences obtained from controls ($p=0.068$). In addition, precore mutation G1896A was detected in

Table 2
Clinical and serological characteristics of patients at baseline and at time of reactivation.

Patient no.	Year of reactivation	Gender/ age	Type of cancer	HBV status at baseline				HBV status at time of reactivation				Treatment	Outcome
				HBsAg	HBsAb	HBeAg	HBV DNA	HBsAg	HBsAb	HBeAg	HBV DNA (IU/mL)		
1	2002	F/76	MH	Neg	28	N.a.	N.a.	Pos	Neg	Neg	6,963,350	LAM	Death
2	2004	M/63	MM	**Neg**	**58**	**Neg**	**Pos (27 IU/mL)**	**Neg**[a]	**Neg**	**Neg**	**42,150**	LAM+ADV	Resolved
3	2005	M/70	NHL	Neg	Neg	N.a.	N.a.	Pos	Neg	Pos	>110,000,000	LAM	Death
4	2005	M/68	NHL	Neg	31	N.a.	N.a.	Pos	Neg	Pos	113,490,000	LAM	Resolved
5	2005	M/54	MM	**Neg**	**15**	**N.a.**	**Pos (164 IU/ mL)**	Pos	Neg	Neg	2790	LAM	Death
6	2005	M/44	AL	Neg	Neg	N.a.	N.a.	**Neg**	**Neg**	**Neg**	**50,118**	LAM	Resolved
7	2005	M/67	NHL	Neg	81	N.a.	N.a.	Pos	Neg	Neg	3981	LAM	Resolved
8	2005	M/54	NHL	**Neg**	**Neg**	**N.a.**	**Pos**[b]	Pos	Neg	Neg	78,800,000	LAM	Resolved
9	2006	M/65	NHL	Neg	Neg	/	N.a.	Pos	Neg	Neg	81,000,000	None	Death
10	2008	M/61	NHL	**Neg**	**Neg**	**N.a.**	**Pos (30 IU/mL)**	**Neg**	**535**	**Neg**	**5780**	TDF	Resolved
11	2007	M/48	NHL	**Neg**[a]	**Neg**	**Neg**	**N.a.**	Pos	Neg	Neg	>110,000,000	ETV	Resolved
12	2009	M/59	NHL	Neg	14	Neg	Neg	**Pos**	**Pos**	**Neg**	**480,000**	TDF	Resolved
13	2009	M/79	NHL	Neg	897	N.a.	N.a.	Pos	Neg	Neg	128,901	ETV	Resolved
14	2009	M/72	LC	**Neg**	**Neg**	**N.a.**	**Pos**[b]	Pos	Neg	Pos	167,792	ETV	Resolved
15	2010	M/65	MM	Neg	594	N.a.	Neg	Pos	Neg	Pos	83,000,000	TDF	Resolved
16	2010	M/66	CLL	Neg	NA	N.a.	N.a.	**Pos**	**154**	**Neg**	**278,535**	ETV	Resolved

Bold font indicates an atypical virological pattern.

[a] Negative result retrospectively found positive with another test.

[b] Not quantifiable.

Table 3
Core/precore mutations and genotype of HBV-R patients at time of reactivation.

		Patients															
		1[a]	**2**	**3**[a]	**4**	**5**[a]	**6**	**7**	**8**	**9**[a]	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Genotype		A	D	D	C	D	D	C	D	D	D	D	D	D	D	D	D
Core/precore positions	**Reference**																
1858	**C**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	C	**T**	C	**T**	**T**	**T**
1896	**G**	**T**	**A**	**A**	G	G	**A**	**A**	**A**	**A**	G	**T**	**A**	**T**	G	G	G
1899	**G**	G	G	**A/G**	**A**	**A**	G	G	G	**A/G**	G	G	**A**	G	G	G	**A**
1762	**A**	A	A	**A/C**	A	A	A	A	**C**	A	A	A	A	A	A	A	A
1764	**G**	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Bold font shows mutated positions.

[a] Patients who died following HBV-R.

HBV collected from 7 out of 16 patients and mutation G1899A was detected in HBV from 6 patients. These frequencies were not significantly different as compared to those observed for control-patients. Interestingly mutation G1899A, previously reported in HBV-related fulminant hepatic failure, was found in 3 out of 4 patients (75%) who died as compared to 3 out of 12 patients (25%) who survived ($p=0.12$) (Alexopoulou et al., 2006).

Amino acid variability within HBsAg

Amino acid variability within HBsAg is represented in Table 4 and Fig. 1. The mean number of substitutions/100 amino acids was significantly higher in the «reactivation» group as compared to the reference group within HBsAg major hydrophilic region (MHR) (7.4 vs. 2.1; $p<10–3$), its «a» determinant (11.5 vs. 2.5; $p=0.003$), and regions 1 (8.3 vs. 2.7; $p=1.7e–3$) and 3 (12.5 vs. 3.1; $p=0.012$) within the HBsAg MHR (Table 3). Significant differences in amino acid variability were also found between HBV-R patients and controls when considering only genotype D sequences from both groups for the MHR (7.4 vs. 2.1; $p<1e–3$) including the "a" determinant (11.7 vs. 3.5; $p<1e–3$), and regions 1 (8.1 vs. 2.6; $p=0.010$) and 3 (12.8 vs. 5.1; $p=0.049$) within HBsAg MHR. Mutations at positions s134 and s175 were the most frequently encountered from patients presenting HBV reactivation, being present in at least 6 of 16 HBV-R patients. Amino acid substitutions at positions s144 (D144A/V) or s145 (G145R/A) within the «a» determinant and well-known to be associated with altered antigenicity of the S protein (http://hbv.geno2pheno.org/index.php) (Carman, 1997; Cooreman et al., 2001; Gerlich, 2006), were found in HBsAg sequences from 7 of 16 patients (44%) with HBV reactivation. Thus, these mutations were significantly more frequently found in the «reactivation» group than in the reference group (25% vs. 0% ($p=0.002$) and 25% vs. 2% ($p=0.01$), respectively). Overall, amino acid substitutions were significantly more frequent at 16 positions within HBsAg in the «reactivation» group as compared to the control group ($p<0.05$; Figs. 1 and 2). In addition, at time of reactivation, all 16 patients presented at least one amino acid substitution within HBsAg among substitutions that were more frequently found in HBV-R than in controls (Fig. 1). In one patient, a deletion corresponding to amino acid 108–114 was found.

Most of HBsAg amino acid substitutions observed here at time of HBV-R were previously reported and several were associated with altered HBsAg immunoreactivity (http://hbv.geno2pheno.org/index.php) (Carman, 1997; Cooreman et al., 2001; Gerlich, 2006; Niscola et al., 2005; Araujo et al., 2009; Weinberger et al.,

Table 4
Number of substitutions per 100 amino acid positions within HBsAg (a) and overlapping regions of reverse transcriptase (b) for HBsAg+/anti-HBsAb+ patients and controls.

a.

Regions of HBsAg	Amino acid positions	Substitutions per 100 amino acid positions within HBsAg ± SD					
		All genotypes			Genotypes D		
		HBV reactivation group (*n*=16)	Control group (*n*=51)	*P*	HBV reactivation group (*n*=13)	Control group (*n*=18)	*P*
N-terminal region	1–99	2.5 ± 4.9	1.9 ± 3.8	0.34	2.6 ± 4.9	1.1 ± 3.4	0.77
MHR (aa 100–169)	100–169	7.4 ± 8.2	2.1 ± 3.2	**1.4e–6**	7.4 ± 8.4	2.1 ± 4.7	**0.9e–5**
"a" determinant*	125–146	11.5 ± 10.9	2.5 ± 3.5	**0.6e–3**	11.7 ± 10.7	3.5 ± 6.7	**0.4e–3**
Region 1	100–120	8.3 ± 6.9	2.7 ± 3.2	**1.7e–3**	8.1 ± 7.9	2.6 ± 4.5	**0.008**
Region 2	122–123	9.4 ± 4.4	6.9 ± 9.7		11.5 ± 5.4	2.8 ± 3.9	0.21
Region 3	125–136	12.5 ± 11.3	3.1 ± 4.0	**0.012**	12.8 ± 10.0	5.1 ± 8.0	**0.049**
Region 4	140–146	9.8 ± 10.7	1.4 ± 2.2	0.064	9.9 ± 12.3	0.8 ± 2.1	0.078
Region 5	148–169	4.8 ± 5.8	1.3 ± 2.1	0.062	4.5 ± 5.6	1.0 ± 2.8	0.066
C terminal region	170–226	6.6 ± 9.1	3.8 ± 7.3	0.073	6.9 ± 9.8	3.7 ± 8.2	0.061
b.							
N–terminal region	1–99	1.6 ± 5.2	1.3 ± 6.1	0.71	1.6 ± 5.2	0.9 ± 4.0	0.29
MHR (aa 100–169)	100–169	5.0 ± 8.0	3.3 ± 6.3	0.16	4.9 ± 8.0	3.5 ± 6.5	**0.03**
"a" determinant**	125–146	8.9 ± 10.7	4.1 ± 7.4	0.09	8.9 ± 11.3	5.3 ± 8.4	0.24
Region 1	100–120	6.8 ± 7.9	6.3 ± 7.9	0.84	6.2 ± 6.7	5.8 ± 7.3	0.85
Region 2	122–123	3.1 ± 4.4	6.3 ± 3.1		3.8 ± 5.4	8.3 ± 3.9	
Region 3	125–136	9.4 ± 11.1	2.7 ± 4.1	0.063	9.6 ± 11.9	4.6 ± 8.1	0.24
Region 4	140–146	8.0 ± 10.7	6.5 ± 11.1	0.801	7.7 ± 10.9	6.3 ± 9.3	0.800
Region 5	148–169	0.9 ± 2.9	0.3 ± 0.7	0.493	1.0 ± 3.6	0.3 ± 1.2	0.53
C terminal region	170–226	1.7 ± 3.7	1.5 ± 3.6	0.77	2.0 ± 4.5	1.1 ± 2.9	0.21

Amino acid variability was analyzed for HBV sequences from any genotype and, separately, from genotype D.
HBsAg, HBs antigen; anti-HBsAb, anti-HBs antibodies; aa, amino acid; MHR, major hydrophilic region; RT, reverse transcriptase; SD, standard deviation.
For analysis, HBsAg was divided into subregions corresponding to structural and/or functional domains: the N terminal region (amino acid 1–99), the major hydrophilic region (MHR; amino acid 100–169) and the C terminal region (amino acid 170–226). The MHR includes five subregions: HBsAg subregion 1 (amino acid 100–120), HBsAg subregion 2 (amino acid 121–123), HBsAg subregion 3 (amino acid 124–137), HBsAg subregion 4 (amino acid 138–147) and HBsAg subregion 5 (amino acid 148–169) (Westhoff et al., 2003). The "a" determinant (amino acid 124–147) spans HBsAg subregions 3 and 4.

2000; Yong-Lin et al., 2012; Simon et al., 2013; Yao et al., 2013; Pezzano et al., 2011; Baclig et al., 2014; Lee et al., 2005; Moradi et al., 2012; Lin et al., 2013; Salpini et al., 2015). In contrast, two substitutions N131A observed here in a patient whose serum sample tested HBsAg-negative at time of HBV-R (pt 10) and S136F (pt 5) were not, to our knowledge, previously described. Moreover, additional N-linked glycosylation sites were detected within HBsAg MHR from HBV of genotype D obtained from two patients with HBV-R, due to amino acid substitutions T116N (in pt 3) and Y134N (pt 10), and the latter was associated with HBsAg negativity at time of HBV-R. In HBV sequences from control patients, an additional N-linked glycosylation site was detected in two patients infected with HBV of genotype A in association with amino acid substitution T131N and one patient infected with HBV of genotype D in association with amino acid substitution M133T.

In 4 patients (pts 2, 8, 10 and 14), HBsAg sequences could be compared before CT and at time of reactivation (Table 5). In one patient (pt 8), although several HBsAg HBV mutants could be detected before CT, a major HBsAg mutation at position 144 could only be detected at time of reactivation. In the 3 other patients (pts 2, 10 and 14), HBV obtained before CT were almost identical to those obtained at time of diagnosis of HBV reactivation, including the presence of major HBsAg mutations in 2 case-patients (G145R in patient 2 and D144A in patient 10).

Amino acid variability within HBV reverse transcriptase

Amino acid variability of HBV-RT is represented in Fig. 2. Regions of RT overlapping the previously defined sub-regions of HBsAg were separately analyzed to appreciate the potential impact of HBsAg variability on RT variability.

The mean number of substitution per 100 amino acids within the RT region corresponding to the HBsAg "a" determinant was higher in the «reactivation» group, although the difference did not reach significance (8 vs. 3.6; $p=0.12$). We paid particular attention to the RT region that overlaps the C terminal region of HBsAg and spans sub-domains B, C and D of HBV RT, within which mutations described as conferring resistance to antiviral drugs occur (Locarnini and Bowden, 2010). In this RT region, the mean number of substitution per 100 amino acids was not different between the «reactivation» group and the control group (1.3 vs. 1.3).

HBsAg subregions	HBsAg position	HBsAg amino acid	HBV reference sequences	Patient identification																Variability (%)		P
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Reactivation group	Control group	
			Genotypes of HBV sequences																			
			A C D	A	D	D	C	D	D	C	D	D	D	D	D		D	D	D			
N-terminal	2	E	- - -	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	6.3	0.0	
	3	N	- - -	-	-	-	N	-	S	-	-	-	-	-	-	S	-	-	-	18.8	4.1	0.09
	4	I	- - -	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	6.3	0.0	
	8	F	- - -	-	-	-	L	-	-	-	-	-	-	-	-	P	-	-	L	18.8	2.0	**0.043**
	10	G	- - -	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	6.3	2.0	
	11	P	- - -	-	-	-	-	-	-	-	-	-	-	-	PS	-	-	-	-	6.3	0.0	
	19	F	- - -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	6.3	4.1	
	24	R	- - -	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	K	12.5	2.0	
	28	I	- - -	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	6.3	2.0	
	40	N	- - -	-	-	-	-	-	-	-	-	-	-	NS	-	S	-	-	-	12.5	23.5	
	41	F	- - -	-	-	-	-	-	-	-	-	-	-	FS	-	-	-	-	-	6.3	0.0	
	44	G	- - -	-	-	-	-	-	-	-	-	-	-	EG	-	-	-	-	-	6.3	11.8	
	45	S	- T T	-	-	N	-	A	-	-	-	-	-	-	-	-	-	-	-	12.5	23.5	
	46	P	- T T	-	-	-	-	P	-	-	-	TA	-	-	-	-	-	-	-	12.5	3.9	
	47	V	- - -	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	6.3	2.0	
	49	L	- - -	R	-	R	-	P	-	-	-	-	-	-	-	-	-	-	-	18.8	7.8	
	53	S	- - -	-	-	-	L	-	L	L	-	-	-	-	-	-	-	-	-	18.8	3.9	0.08
	55	S	- - -	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	6.3	5.9	
	57	T	- - -	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	6.3	7.8	
	68	I	- T T	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	6.3	7.8	
	86	I	- - -	-	-	-	-	IT	-	-	-	-	-	-	-	-	-	-	-	6.3	2.0	
	92	I	- - -	-	-	-	-	IT	-	-	-	-	-	-	-	-	-	-	-	6.3	2.0	
	93	F	- - -	-	-	-	-	FS	-	-	-	-	-	-	-	-	-	-	-	6.3	3.9	
	94	L	- - -	-	-	-	-	-	-	-	-	LS	-	-	S	-	-	-	-	12.5	2.0	0.139
	96	V	- - -	-	-	-	-	VG	-	-	-	VG	-	-	-	-	-	-	-	12.5	2.0	0.139
MHR-1	100	Y	- - -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	6.3	5.9	
	101	Q	- - -	-	H	-	-	-	-	-	-	-	R	-	-	-	H	-	-	18.8	3.9	0.08
	102	G	- - -	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	6.3	2.0	
	103	M	- - -	-	-	I	I	-	-	-	-	-	-	-	-	-	-	-	-	12.5	0.0	**0.05**
	108	P	- - -	-	-	-	-	-	-	-	-	-	Ind	-	-	-	-	-	-	6.3	0.0	
	109	L	- - -	-	-	-	-	-	-	-	-	-	Del	-	-	I	Q	-	-	18.8	2.0	**0.039**
	110	I	- L -	-	-	-	-	-	L	-	-	-	Del	-	-	-	-	-	-	12.5	13.7	
	111	P	L/- - -	-	-	-	-	-	-	-	-	-	Del	-	-	-	-	-	-	6.3	0.0	
	112	G	- - -	-	-	-	-	-	-	-	-	-	Del	-	-	-	-	-	-	6.3	0.0	
	113	S	- T -	-	-	-	-	-	T	-	-	-	Del	-	-	-	-	-	-	12.5	2.0	0.13
	114	T	- S S	-	-	-	A	SP	-	-	-	-	Ind	-	-	-	-	-	-	18.8	5.9	0.14
	116	T	- - -	-	-	N	-	NT	-	-	-	-	-	-	-	-	-	-	-	12.5	0.0	**0.05**
	118	T	- - -	-	-	-	R	-	-	K	-	-	-	-	-	-	-	-	A	18.8	3.9	0.08
	119	G	- - -	-	-	-	-	-	-	AE	-	-	-	-	-	-	-	-	-	6.3	3.9	
	120	P	- - -	-	-	-	-	PS	-	-	-	-	A	-	-	-	-	-	-	12.5	3.9	
MHR-2	122	K	- R/- R	-	-	-	-	-	-	-	-	KR	H	-	-	-	-	-	-	12.5	13.7	
	123	T	- - -	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.3	0.0	

HBsAg subregions	HBsAg position	HBsAg amino acid	HBV reference sequences	Patient identification																Variability (%)		P
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Reactivation group	Control group	
			Genotypes of HBV sequences																			
			A C D	A	D	D	C	D	D	C	D	D	D	D	D		D	D	D			
MHR-3	125	T	- - -	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	6.3	5.9	
	126	T	- I -	-	-	-	T	-	I	T	-	TN	-	-	-	-	-	-	-	**25.0**	**0.0**	**0.002**
	127	P	- - -	-	-	-	-	PLIT	-	-	T	-	H	-	-	-	-	T	-	25.0	11.8	0.18
	129	Q	- - -	-	-	-	-	-	-	-	-	QRL	-	-	-	-	-	-	-	6.3	0.0	
	130	G	- - -	-	-	-	-	-	-	-	-	GNKSRDE	-	-	-	-	-	-	-	6.3	2.0	
	131	N	- T T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	N	12.5	3.9	
	133	M	- - -	-	-	-	-	-	-	-	-	ML	-	-	-	-	-	-	I	12.5	5.9	
	134	F	- - Y	-	-	-	I	-	F	Y	-	YN	N	-	-	N	-	-	-	**37.5**	**7.8**	**0.009**
	135	P	- - -	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	6.3	0.0	
	136	S	- - -	-	Y	-	-	SF	-	-	-	-	-	-	-	-	-	-	-	**12.5**	**0.0**	**0.05**
MHR-4	140	T	- - -	-	-	-	-	-	-	-	TI	-	-	-	-	-	-	-	-	6.3	0.0	
	142	P	- - -	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	6.3	2.0	
	143	T	- S S	-	-	-	-	-	-	-	ST	-	-	-	-	-	-	-	-	6.3	5.9	
	144	D	- - -	-	-	-	-	DA	-	-	DV	-	A	-	E	-	-	-	-	**25.0**	**0.0**	**0.002**
	145	G	- - -	-	R	-	A	-	-	-	-	GR	-	-	GE	-	-	-	-	**25.0**	**2.0**	**0.01**
	149	C	- - -	-	-	-	-	-	-	-	CF	-	-	-	-	-	-	-	-	6.3	0.0	
	156	W	- - -	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	6.3	0.0	
	158	F	- - -	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	6.3	3.9	
	159	A	- - G	-	-	-	-	-	A	-	-	GA	-	-	-	-	-	-	-	12.5	7.8	
MHR-5	160	K	- R -	KR	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	**12.5**	**0.0**	**0.05**
	161	Y	- F F	-	-	-	-	-	-	-	-	FY	-	FY	-	-	-	-	-	12.5	5.9	
	164	E	- - -	EG	-	-	-	-	-	-	-	-	G	-	-	-	V	-	-	**18.8**	**0.0**	**0.011**
	165	W	- - -	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	6.3	2.0	
	166	A	- - -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	6.3	2.0	
	168	V	- - A	-	-	-	A	-	V	-	-	-	-	-	-	-	-	-	-	12.5	2.0	0.139
	169	R	- - -	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	6.3	0.0	
	170	F	- - -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	6.3	0.0	
	172	W	- - -	-	-	-	-	WL	-	-	-	-	-	-	C	-	-	-	C	**18.8**	**0.0**	**0.011**
	174	S	- - -	-	N	-	N	-	-	-	-	-	-	-	-	N	-	-	-	**18.8**	**0.0**	**0.011**
	175	L	- - -	LS	S	-	-	-	-	-	LS	-	S	-	S	-	-	-	S	**37.5**	**3.9**	**0.0016**
	177	V	- - -	-	-	-	A	-	-	-	-	VL	-	-	A	A	-	-	-	**25.0**	**2.0**	**0.0010**
	181	Q	- - -	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	6.3	2.0	
	183	F	- - -	-	-	-	-	-	-	-	FL	-	-	-	-	-	-	-	-	6.3	0.0	
	184	V	-/1 -	-	-	-	-	-	-	-	-	-	-	AV	-	-	-	-	-	6.3	2.0	
	185	G	- - -	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	6.3	0.0	
	190	V	- - -	-	-	-	-	VA	-	-	-	-	-	-	-	-	-	-	-	6.3	5.9	
	193	S	- -/L	-	-	L	-	LS	-	-	-	-	-	LS	-	-	-	-	S	**25.0**	**2.0**	**0.010**
C-terminal	194	A	- V V	-	-	-	V	-	-	V	-	VA	-	-	-	-	-	-	-	**18.8**	**15.7**	
	197	M	- - -	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	**6.3**	**3.9**	
	202	G	- - -	-	-	-	-	-	-	-	-	-	-	-	-	GE	-	-	-	**6.3**	**0.0**	
	203	P	- - -	-	-	-	-	-	-	-	-	-	-	LP	-	-	-	-	-	**6.3**	**2.0**	
	204	S	- - -	-	-	-	-	SN	-	-	K	-	-	GRS	-	-	-	-	-	**18.8**	**25.5**	
	206	Y	- - -	-	-	-	-	-	-	-	C	-	-	CY	C	-	-	-	-	**18.8**	**15.7**	
	207	S	- - -	-	-	-	-	-	N	-	T	-	-	HNPRST	R	R	-	-	-	**31.3**	**39.2**	
	208	I	- - -	-	-	-	-	-	-	-	-	IT	-	IT	-	-	-	-	-	**12.5**	**7.8**	
	209	V	- L L	-	-	-	-	-	-	-	-	LV	-	-	-	-	-	-	-	**6.3**	**0.0**	
	210	S	- - -	-	-	-	N	-	N	N	-	SR	-	-	-	-	-	-	-	**25.0**	**25.5**	
	211	P	S - -	-	-	-	-	-	-	-	PH	-	-	-	-	-	-	-	-	**6.3**	**3.9**	
	213	I	- L L	-	-	I	-	-	-	-	-	-	S	-	-	-	-	-	-	**12.5**	**7.8**	
	214	P	- - -	-	-	-	-	-	-	-	-	-	-	LP	-	-	-	-	-	**6.3**	**7.8**	
	216	L	- - -	-	-	stop	-	FL	-	-	-	-	-	-	-	-	-	-	-	**12.5**	**7.8**	
	217	P	- - -	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	**6.3**	**3.9**	
	220	F	- - -	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	**6.3**	**2.0**	
	221	C	- - -	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	**6.3**	**0.0**	
	226	I	- - -	-	-	-	-	-	-	IT	-	-	-	-	-	-	-	-	-	**6.3**	**0.0**	

Fig. 1. Amino acid variability in the hepatitis B surface antigen from patients with and without hepatitis B reactivation.
Amino acid variability was analyzed for HBV sequences from any genotype and, separately, from genotype D. Both amino acids are noted when two different amino acids are harbored at the same position by reference sequences of the same genotype. Proportion of mutated sequences at a given position was calculated as follows: proportion of sequences that do not harbor amino acid(s) found in HBV reference sequences of the same genotype. Amino acids harbored by HBV genotypes A, C, D from the NCBI HBV genotyping reference panel (http://www.ncbi.nih.gov/projects/genotyping/view.cgi?db=2) are shown on the left side. For HBV reference sequences: only positions with amino acid substitutions as compared to HBV reference sequences of same genotypes are shown; a dash means the absence of amino acid substitution. For HBV sequences from patients: only positions with amino acid substitutions in HBsAg as compared to HBV reference sequences of corresponding genotype are shown; a dash means the absence of amino acid substitution. *P* value is indicated by a boldface at positions where the proportion of sequences harboring mutations was significantly higher from patients with hepatitis B reactivation than from controls are marked with an asterisk; when wild-type and mutated amino acid(s) are concomitantly found at a given position in bulk sequence, all these amino acids are indicated; indeed, our direct sequencing method allows the detection of mixed sequences from different HBV quasi-species. Amino acid substitutions found in HBV sequences from patients with HBV reactivation and not from control patients are in a grey font and framed.

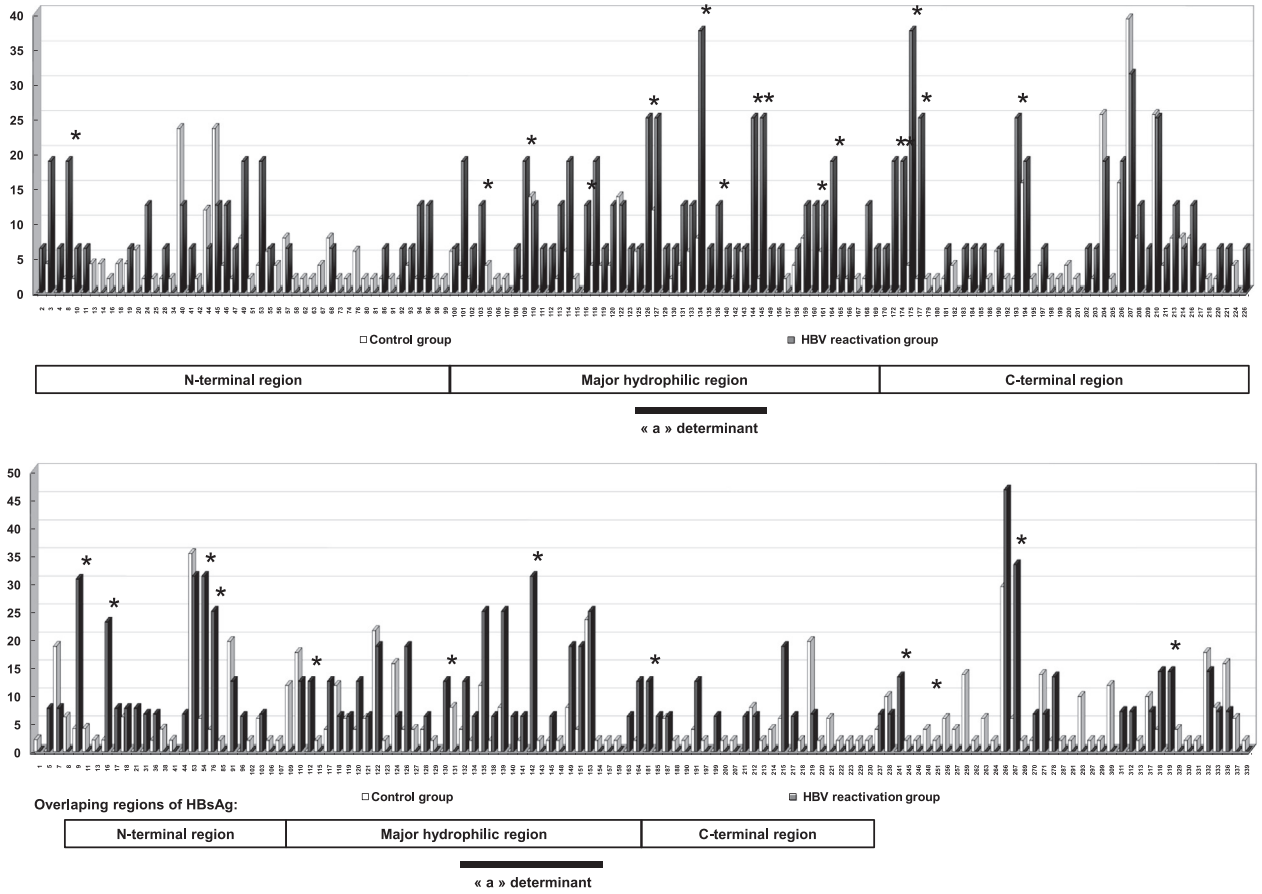

Fig. 2. Amino acid variability within HBsAg (a) or reverse transcriptase (b) of HBV sequences from patients with HBV reactivation (dark grey rods) or control patients (white rods). Amino acid variability was considered the percentage of sequences that harbored at a given position another amino acid that the one found in HBV reference sequences of the same genotype (found in the genotyping reference set of HBV sequences available on the NCBI Web site, http://www.ncbi.nih.gov/projects/genotyping/view.cgi?db=2). Only variable positions (in HBV from either the case-patients or the control-patients, or from patients of both groups) are shown.

Table 5
HBsAg amino acid substitution before and at time of HBV reactivation in the four patients in whom HBV DNA could be sequenced before reactivation.

Patient no.	HBsAg amino acid substitutions at positions where variability was significantly higher in HBV from patients presenting reactivation than from controls		Amino acid substitutions within HBsAg between sequences obtained before and at time of reactivation
	Before reactivation	At time of reactivation	Before reactivation/at time of reactivation
2	M103M/I, S136S/Y, G145R, S174N, L175L/S	S136/Y, G145R, S174N, L175S	M/I103M, Y/S136Y, C/W137C, S/L175S, T/I226T
8	F8L, E164D, S193L	D144D/V, L175L/S	K/R24K, S/N31S, P/T67P, A118T, G/R130G, T140T/I, D144D/V, G/A159G, D164E, S/L167S, L175L/S, T181Q, G/V182W, A186L, F187S, P189T, L193S, W/R196W, Y/C200Y, G/V202G, G/C206C, T/I207T, P211P/H, P/Q217P, F/C220F
10	Y134N, D144A, E164G, L175S	Y134N, D144A, E164G, L175S	Q129P, G130E, L213S
14	E164V	E164V	None

Nonetheless, amino acid substitutions within HBV-RT were more frequently found in the «reactivation» group than in the control group at 11 different amino acid positions (rt9, rt16, rt54, rt76, rt112, rt130, rt142, rt181, rt241, rt267 and rt319) including positions rt181 and rt241 located within the D domain of HBV RT. Amino acid substitution at position rt181, known to confer resistance to lamivudine and adefovir, could be detected from 2 patients (pts 12 and 16) at time of reactivation, before introduction of any antiviral drugs. This substitution was never detected in HBV from patients in the control group ($p=0.05$). Moreover, amino acid rt153Q, corresponding to s145R, was found in HBV from 5 patients. This amino acid substitution rtR153Q is known to restore HBV replication of LAM resistant mutants (Carman, 1997).

Finally, a 12-nucleotide deletion within RT coding region corresponding to amino acid positions 118–121 was found in 27% of HBV clonal sequences from pt 10.

Discussion

In the present work, we show that specific genotypic HBV patterns are found in patients presenting with HBV-R following

chemotherapy for cancer. We found that in the context of chemotherapy, HBV-R involved more frequently non-A HBV genotypes (mostly D genotypes) than genotype A viruses, although genotype A is the most frequently found in our area (Tamalet et al., 2006). This finding is congruent with the results from Salpini et al. (2015) who recently reported that all 29 patients presenting HBV-R were infected with HBV of genotype D, and it adds further data to previously published results suggesting that specific clinical outcomes of chronic hepatitis B might be associated with specific HBV genotypes (Malmström et al., 2012; Lin and Kao, 2011). We also observed a considerable frequency of core and/or precore mutations in viruses from patients presenting HBV reactivation. Thus, core/precore HBV mutants could be detected from all patients. The high frequency of these mutants might simply reflect the fact that most patients (86%) were infected with genotype D HBV strains, which are known to favor the emergence of core/precore mutants (Malmström et al., 2012). These data suggest that core/precore mutants are likely either to promote HBV reactivation or to be specifically selected during HBV reactivation. Emergence of core/precore mutants has been previously reported in the context of HBV reactivation (Alexopoulou et al., 2006; Gérolami et al., 2005). Core/precore mutations have been described to confer higher replicative capacity to HBV (Yeo et al., 2000; Ozasa et al., 2006). In the absence of immune system pressure following polychemotherapy, these HBV strains could therefore be more likely to emerge as major strains. In addition, these viruses might also be involved in the severe clinical outcome of HBV reactivation (Sainokami et al., 2007). Indeed, mutation G1899A, previously reported being associated with HBV-related fulminant hepatic failure, was three-times more frequent in the present study in HBV from patients who died from fulminant hepatic failure than from those who survived (Sainokami et al., 2007).

An important result of the present work is the high frequency of amino acid substitutions that could be detected within HBsAg in HBV from patients with HBV reactivation.

Detection of HBsAg mutants has been described in up to 11% of chronic HBV carriers in Asia and has been reported to be frequently associated with different HBV clinical contexts including following HBV vaccine immunization, nucleoside analog therapy or anti-HBsAg immunoglobulin injection (see for review Alavian et al. (2013)). The detection of such HBV mutants in the context of HBV-R has been previously described in some case reports or series (Seto et al., 2014; Alexopoulou et al., 2006; Westhoff et al., 2003). In the recent study from Salpini et al., HBsAg substitutions were detected in 22 out of the 29 patients who developed HBV-R during immunosuppression, and 13 of these substitutions were significantly more frequent in patients presenting HBV-R than in controls. It has to be underlined that HBsAg was positive in 10 of these 29 patients before immunosuppressive treatment (Salpini et al., 2015). Noteworthy, in this study, prevalence of HBsAg amino acid substitutions D144E and G145R known to hamper HBsAg recognition by anti-HBsAb was 21% and 17% in patients with HBV-R, respectively, being significantly more frequent than in control-patients. In addition, in the study from Seto et al. (2014), substitution G145R was detected in one out of four patients in whom HBsAg could be sequenced. We also previously reported the detection of HBsAg amino acid substitutions in 7 patients presenting with HBV reactivation from a cohort of 51 HBsAg-negative/anti-HBc-positive patients who underwent chemotherapy for cancer (Borentain et al., 2010). However, in this latter study, the small number of patients and the absence of control group prevented from drawing conclusion on the meaning of this finding. The present study, extended to a larger group of 16 consecutive patients and a large control group of chronic HBV carriers, strongly suggests that mutations within HBsAg are systematically encountered in patients presenting HBV reactivation. This finding has important clinical implication because HBsAg HBV mutants have been associated with false negativity of HBsAg serological testing as well as peculiar HBV serological patterns such as the concurrent detection of HBsAg and anti-HBsAb (Colson et al., 2007). Noteworthy, in the present study, unusual HBV serological patterns were found in 5 patients (31%) at time of reactivation. These confusing serological results may delay the diagnosis and therefore the treatment of HBV reactivation. This is a critical issue as HBV reactivation is a life threatening disease (25% mortality rate in our study) and early treatment might be mandatory to improve the prognosis. In view of the impaired performance of HBV serological testing in these patients, HBV DNA detection should be performed in patients presenting with acute hepatitis in a context of chemotherapy for cancer, regardless of the results of HBV serology.

The significance of the emergence of HBsAg HBV mutants in the context of chemotherapy for cancer is unknown. We have previously reported that peculiar serological patterns such as concurrent detection of HBsAg and antiHBs-Ab, associated with HBsAg mutations, were frequently observed in immunocompromized patients (Colson et al., 2007). Patients undergoing chemotherapy for cancer, especially hematological cancer, or patients undergoing specific therapies such as rituximab have impaired immunity. In these patients, the decreased affinity of anti-HBsAb for mutated HBsAg might lower their protective effect and decrease the overall control of HBV replication by the immune system. In their recent study, Salpini et al. (2015) identified additional N-linked glycosylation sites related to HBsAg mutations within the major hydrophilic region that can mask immunogenic epitopes. We detected such additional N-linked glycosylation sites from two patients infected with HBV of genotype D. Therefore, HBsAg mutated HBV strains might be positively selected following immunosuppression in patients who have previously developed efficient humoral immune response against HBV.

Another explanation for the high frequency of HBsAg mutations in HBV-R patients might be that chronic HBV carriers harboring HBsAg mutations might have been misdiagnosed as HBsAg-negative by serological testing at baseline. Indeed, it is striking that detection of HBV DNA despite negative HBsAg testing could be demonstrated in 5 out of the 7 patients tested at baseline, while in another patient, HBsAg was retrospectively found positive before chemotherapy initiation using a second serological assay. In this view, our study also raises the question of the optimal HBV monitoring that should be proposed to patients. To date, preventive treatment of HBsAg-positive patients before chemotherapy is recommended (European Association For The Study Of The Liver, 2012). Regarding HBsAg-negative patients, HBV monitoring, including HBV DNA detection at time of HBV serological screening, has been increasingly proposed in patients presenting with isolated anti-HBcAb before chemotherapy (European Association For The Study Of The Liver, 2012; Hwang and Lok, 2014). Thus, the risk of HBV reactivation in isolated anti-HBcAb positive patients undergoing chemotherapy for lymphoma has been reported to be as high as 25–30% (Hsu et al., 2014; Mikulska et al., 2014).

Recommendations for patients presenting with a serological pattern of past “cured” HBV infection (as indicated by concurrent detection of anti-HBc and anti-HBs antibodies) are still debated. Indeed, the risk of HBV reactivation following chemotherapy is not well known in these patients. In our previous study, 3 out of 56 patients (5.3%) positive for both anti-HBc and anti-HBs antibodies developed HBV reactivation following chemotherapy. It is however worthy to note that, in the present study, 8 of 15 tested patients (56%) were positive for anti-HBsAb with a significant titre (up to 897 IU/mL) at baseline.

In the recent study from Taiwan by Hsu et al. (2014), 53% of lymphoma patients presenting HBV reactivation were antiHBsAb-positive before chemotherapy initiation. The proportion of antiHBsAb-positive patients at baseline was 68% in the study from Seto et al. (2014) and 64% in the study from Mikulska et al. (2014).

These findings suggest that in the context of chemotherapy for cancer, detection of anti-HBsAb does not exclude the possibility of low-level HBV replication and strongly support the use of HBV DNA testing before chemotherapy in anti-HBcAb positive patients, regardless the results of anti-HBsAb testing (Ferraro et al., 2009). In this view, in our present study, two antiHBsAb positive patients, out of the four tested, were retrospectively found positive for HBV DNA testing before chemotherapy initiation.

Another important consequence of HBsAg variability is its possible impact on HBV reverse transcriptase (Torresi et al., 2002). We show that HBVrt from patients undergoing HBV reactivation display an increased variability as compared to HBVrt from newly diagnosed HBV-infected patients. Moreover, amino acid substitution at position rt181 could be found in HBV strains from 2 patients before any treatment. Substitution at this position has been shown to confer resistance of HBV strains to lamivudine as well as adefovir (Gerolami et al., 2006). Although most published studies used lamivudine and lamivudine is still proposed in the preemptive or prophylactic treatment of HBV reactivation following chemotherapy (European Association For The Study Of The Liver, 2012), the present data add further evidence that the use of drugs with a high genetic barrier for resistance (tenofovir and entecavir) should be favored in the clinical context of HBV reactivation (Seto et al., 2014; Salpini et al., 2015; Colson et al., 2008; Nakamoto et al., 2014; Kim et al., 2012; Li et al., 2011). In this view, it is noteworthy that in the present work, all deaths occurred between 2002 and 2006 (i.e. before introduction of entecavir and tenofovir) ($p < 0.05$). This might reflect a global improvement in the management of these patients, including an earlier diagnosis and treatment, but also a more efficient therapy. Thus, none of the deceased patients had received entecavir or tenofovir treatment.

In conclusion, we show that HBV reactivation following chemotherapy in patients testing negative for HBsAg before chemotherapy may occur in patients presenting with isolated anti-HBcAb but also in those positive for anti-HBsAb. In this context, HBV reactivation is associated with an important genetic variability of HBV strains within HBsAg and HBVrt. The emergence of such mutated HBV strains is associated in these patients with impaired serological diagnosis of HBV reactivation as well as an increased risk of emergence of HBV strains resistant to antiviral drugs. These data strongly support the use of HBV DNA detection in the management of these patients before and during chemotherapy and the use of antiviral drugs with a high genetic barrier to resistance in case of HBV reactivation.

Material and methods

Patients

From July 2002 through December 2010, 22 cases of HBV-related acute hepatitis in a context of polychemotherapy were recorded in Marseille university hospitals. In 4 cases, HBV serological status before initiation of anticancer treatment was not known. In 2 cases, the patients were known to be HBsAg-positive and HBV-related acute hepatitis occurred in the absence of preemptive antiviral treatment (one case) or after interruption of anti-HBV therapy (one case). In the remaining 16 cases, HBV serological status at time of initiation of chemotherapy was indicative of past-HBV infection, i.e. showed anti-HBc antibody-positivity but HBsAg-negativity, with or without anti-HBsAb. These 16 patients were selected for the study (Tables 1 and 2). The research was conducted on plasma samples collected at the time of diagnosis of HBV reactivation. Nine of these patients were previously partially described (Borentain et al., 2010; Gérolami et al., 2005, 2007). For comparative analysis of virological patterns including HBsAg and RT regions, a control group consisting of 123 individuals with newly diagnosed chronic HBV infection over a 12-month period (May 2003–April 2004) was used. Serum samples had been obtained at diagnosis, before any antiviral treatment initiation and HBV sequences have been obtained from 51 of them; among these sequences, 26, 2, 18 and 5 belonged to genotypes A, C, D and E, respectively (Table 1) (Colson et al., 2007).

HBV serological tests and HBV DNA detection

HBV serological markers (HBsAg, anti-HBcAb, and anti-HBsAb (analytical threshold, 10 IU/L)) and anti-HCV antibodies were tested using Axsym Abbott assays (Abbott Diagnostics Division, Wiesbaden, Germany). When possible a second test (Vidas, Bio-Mérieux, Meylan, France) was used for HBsAg determination. Serum HBV DNA determination used the Cobas Ampliprep/Cobas TaqMan assay, the Roche real-time assay (Roche Diagnostics, Meylan, France; detection threshold of 6–30 IU/mL), or the Abbott RealTime assay (Abbott Diagnostics Division, Wiesbaden, Germany; detection threshold of 10 IU/mL).

HBV genotypic and amino acid patterns

Nucleic acid extraction and purification, PCR amplification and DNA sequencing

Whole blood was collected in dry tubes with gel separator. Amplification and direct sequencing of the full-length HBsAg coding-region and the entire RT gene were performed using in-house protocols as previously described (Colson et al., 2007). Sequencing was performed with the BigDye Terminator Cycle sequencing kit v1.1 (Applied-Biosystems, Branchburg, NJ, USA) on the ABI Prism 3130 genetic analyzer (Applied-Biosystems). Nucleotide sequences were aligned using Seqscape software v2.5 (Applied-Biosystems) then compared with a set of sequences available in GenBank for genotype determination using the MEGA software (Kumar et al., 1994).

Analysis of amino acid substitutions within HBsAg and reverse transcriptase

The nucleotide HBsAg/RT sequences obtained were translated into amino acid sequences, aligned and compared with HBV sequences of the same genotype found in the genotyping reference set available on the NCBI website (URL: http://www.ncbi.nih.gov/projects/genotyping/view.cgi?db=2). A list of HBsAg amino acid substitution described as affecting the recognition of HBsAg by anti-HBsAb was obtained from the Geno2pheno [hbv] 2.0 website of the Max-Planck-Institut für Informatik (URL: http://hbv.geno2pheno.org/index.php).

Amino acid variability was defined as the proportion, at each position, of sequences that did not harbor the amino acid found in HBV reference sequences of the same genotype. For analysis, HBsAg was divided into sub-regions corresponding to structural and/or functional domains: the N terminal region (amino acid 1–99), the major hydrophilic region (MHR; amino acid 100–169) that includes the «a» determinant (amino acid 124–147) spanning HBsAg sub-regions 3 and 4, and the C terminal region (amino acid 170–226) (Cooreman et al., 2001; Lin et al., 2001). HBV genotypic patterns from patients presenting HBV reactivation were compared to those obtained from 51 newly diagnosed HBV carriers.

Analysis of N-linked glycosylation sites

N-linked glycosylation sites were predicted along the full-length HBsAg amino acid sequences obtained from the 16 patients

with HBV-R using the N-glycosite tool that is available online through the Los Alamos National Laboratory website (URL: http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html) and detects Nx[ST] patterns (where N=asparagine, S=serine, T=threonine and x can be any amino acid apart from proline) (Zhang et al., 2004).

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